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Published in:
Poultry Science

DOI:
10.3382/ps/pey472

Print publication: 01/09/2019

Document Version
Publisher's PDF, also known as Version of record

IMMUNOLOGY, HEALTH, AND DISEASE

Immune modulation, growth performance, and nutrient retention in broiler chickens fed a blend of phytogenic feed additives

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ABSTRACT This study aimed to assess the effect of a commercial blend of phytogenic feed additives (**PA**), comprising 5% carvacrol, 3% cinnamaldehyde, and 2% capsicum oleoresin on the modulation of immune biomarkers of broiler chickens, their growth performance, dietary energy, and nutrient retention. Four-hundred day-old birds were assigned to one of four dietary treatments. Two control diets based on either wheat (**WC**) or maize (**MC**) were each given with and without PA at 100 g/t. Growth performance variables including feed intake (**FI**), weight gain (**WG**), and feed conversion ratio (**FCR**) were recorded. Dietary N-corrected apparent metabolizable energy (**MEn**), dry matter (**DMR**), nitrogen (**NR**), and fat retention (**FR**) coefficients were also determined. Gene expression of immune biomarkers (cytokines) were determined in caecal tonsil tissue from 21 d old birds. Expression of IL2, IL18, IL10, and IL17C in the caecal tonsils were upregulated ($P < 0.05$) in the birds fed MC-based diets compared to the WC fed birds. Feeding PA supplemented diets downregulated the expression of CD40LG ($P < 0.001$), IFNG, and IL6 ($P < 0.05$). There was a cereal type × PA interaction ($P < 0.05$), as expression of IFNB was downregulated in the birds fed PA supplemented MC but not WC. However, expression of IL12B was downregulated in birds fed PA supplemented WC but there was no significant ($P > 0.05$) change in expression levels in birds fed MC diets. Feeding MC diets gave greater FI ($P < 0.001$) and ME ($P < 0.05$), but lower FCR ($P < 0.05$) compared to birds fed WC diets. The WG and nutrient retention coefficients were not affected ($P > 0.05$) by cereal type. Supplementary PA improved FI ($P < 0.05$), WG ($P < 0.001$), FCR ($P < 0.05$), MEn ($P < 0.05$), MEn: GE ratio ($P < 0.05$), and FR ($P < 0.05$). In conclusion, dietary inclusion of PA improved overall growth performance variables, energy, and nutrient retention and intestinal cytokine expression.

Key words: plant extracts, broiler chickens, immune modulation, metabolizable energy

2019 Poultry Science 98:3443–3449
http://dx.doi.org/10.3382/ps/pey472

INTRODUCTION

Inclusion of phytogenic feed additives (**PA**) in diets aiming to improve performance and health has been promoted for broiler chickens and other farm animals (Windisch et al., 2008; Wallace et al., 2010). Supplementation of PA to broiler diets has been shown to improve growth performance variables (Jamroz et al., 2003; Pirgozliev et al., 2015a,b), dietary available energy, and nutrient digestibility (Mountzouris et al., 2010; Bravo et al., 2011, 2014), as well as improve innate immunity and host disease resistance (Lee et al., 2010, 2013), and antioxidative status (Karadas et al., 2014). The efficiency of dietary PA may also be influenced by the hygienic conditions where birds are reared (Pirgozliev et al., 2014). Addition of PA to animal diets alters normal gut microflora in broiler chickens (Kim et al., 2015), decreasing the prevalence of pathogens, preventing colonization of the gastrointestinal tract (Mitsch et al., 2004; Oviedo-Rondón et al., 2006). There is also increasing evidence that through interactions with the immune system, PA are capable of modulating immune responses (Kim et al., 2015). In the absence of antibiotics to promote animal growth, dietary immunomodulation is a key antibiotic alternative that can contribute to the enhancement of productivity and integrity of the immune system in farm animals (Kumar et al., 2011;

Received May 21, 2018.
Accepted September 12, 2018.
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Munyaka et al., 2012). The present experiment aimed to assess the effect of a commercial blend of PA, including carvacrol, cinnamaldehyde, and capsicum oleoresin on local expression of cytokine biomarker genes in the caecal tonsils that may indicate modulation of the immune response of rapidly growing broiler chickens. In addition, the growth performance, dietary N-corrected apparent metabolizable energy (**MEn**) and nutrient retention were measured to examine the effects of the PA on production variables.

MATERIALS AND METHODS

The Animal Experimental Committee of the Scottish Agricultural College approved all procedures.

Birds Husbandry and Experimental Design

Four-hundred male day-old Ross 308 chicks were obtained from a commercial hatchery and were allocated to 40 floor pens, 10 birds in a pen. Each of the 40 pens had a concrete floor with an area of 2.1 m^2. All birds were placed on litter that was a mixture of approximately 10% new (top-dressed) and 90% used, obtained from a previous crop of broiler chickens reared at the same site. The previously reared flock did not have any clinical health problems. The birds were vaccinated for infectious bronchitis (**IB**) at the hatchery.

Birds were fed one of four diets. There were 2 control diets based on either wheat (**WC**) or maize (**MC**) and were formulated to be nutritionally adequate but marginally lower (about 5%) than the optimum economic ME concentration (Aviagen Ltd, Edinburgh, UK) for male broilers between 0 and 21 d of age (Table 1). A further 2 diets were prepared using the basal control diets supplemented with a commercial blend of PA (XTRACT 6930; Pancosma S.A., Geneva, Switzerland) comprising 5% carvacrol, 3% cinnamaldehyde, and 2% capsicum oleoresin at 100 g/t. The PA was incorporated into the diets in powder form. All the diets were offered in mash form. The diets did not contain any coccidiostat, antimicrobial growth promoters, or similar additives. Feed and water were offered ad libitum to birds throughout the experiment.

Each diet was given to birds in 10 pens following randomization. The experimental room was equipped with a positive pressure ventilation system to meet commercial recommendations. During the study, the temperature was initially 33°C and was gradually reduced to 20°C after birds were 20 d of age. The relative humidity was maintained between 50 and 70%. A standard lighting program for broilers was used, decreasing from 23:1 (hours light: dark) from day old to 18h:6 h at 7 d of age, which was maintained until the end of the study. The feeding period ended when the birds were 21 d of age.

At 21 d of age, one bird from each pen was randomly selected, stunned and killed by cervical dislocation and the left caecal tonsil was collected and stored in RNA*later*® (Sigma-Aldrich, USA) at –80°C prior to analysis on the relative expression of the genes of interest (**GOI**) (Table 2).

Table 1. Ingredient composition of the experimental control diets (as-fed basis).

Ingredients, g/kg	MC	WC
Maize	528.6	–
Wheat	–	546.8
Soybean meal (48)	313.0	274.9
Vegetable oil	10.0	35.0
Barley	63.3	58.4
Rye	50.0	50.0
Dicalcium phosphate	14.3	14.3
Limestone	11.5	11.5
NaCl	3.3	2.7
Lysine HCL	1.5	1.5
Methionine	3.5	3.9
Vitamin mineral premix[1]	1.0	1.0
Total	1000	1000
Calculated analysis (as-fed basis)		
ME, MJ/kg	12.13	12.13
Crude protein, g/kg	215	215
Crude fat, g/kg	34	47
Calcium, g/kg	8.3	8.4
Non-phytate P, g/kg	4.4	4.5
Lysine, g/kg	12.3	12.3
Methionine + Cysteine, g/kg	9.5	9.5
Analyzed values (as fed basis)		
Dry matter, g/kg	864	872
Crude protein, g/kg	197	198
Crude fat, g/kg	35	47

[1]The premix provided (units/kg diet): 12,000 IU retinol, 5000 IU cholecalciferol, 34 mg α-tocopherol, 3 mg menadione, 2 mg thiamine, 7 mg riboflavin, 5 mg pyridoxine, 15 μg cobalamin, 50 mg nicotinic acid, 15 mg pantothenic acid, 1 mg folic acid, 200 μg biotin, 80 mg Fe as iron sulfate (30%), 10 mg Cu as a copper sulfate (25%), 100 mg Mn as manganous oxide (62%), 80 mg Zn as zinc oxide (72%), 1 mg I as calcium iodate (52%), 0.2 mg Se as sodium selenite (4.5%), and 0.5 mg Mo as sodium molybdate (40%).

Total RNA Extraction and Reverse Transcription

The analyses of relative expression of GOI in the caecal tonsils were performed by qStandard (Middlesex, UK). Approximately, 30 mg of macro-dissected caecal tonsil tissue per sample (stored as previously described) was homogenized in 500 μL QIAzol lysis reagent for 10 min at 30 Hz in a Tissuelyzer LT (Qiagen, UK). Lysates were mixed with 100 μL chloroform, transferred to pegGold PhaseTrap tubes (PeqLab, UK) and centrifuged for 5 min in at room temperature. The aqueous phase was poured into fresh tubes, mixed with 1.5 vol of ethanol and applied to Qiagen RNeasy columns (Qiagen, UK). RNA was purified according to the manufacturer's instructions (Qiagen, UK). RNA integrity was assessed using an Agilent Bionalyzer and RIN was > 8 for all samples. Purity and quantity were measured using a NanoDrop spectrophotometer; for all samples the absorbance peak was at 260 nm, A260/280 > 2 and A260/230 > 1. Eight-hundred ng of RNA were reverse transcribed using a Quantitect reverse transcription kit (Qiagen, UK) in a 10 μL reaction according to the manufacturer's instructions. This RT kit includes a

Table 2. RT-qPCR assays[1] for quantification of gene expression in *Gallus gallus* caecal tonsil tissue.

Gene symbol	Gene	Accession number	Primer sequences (5′-3′)	Product length, bp	Location
CD40LG	CD40 ligand	NM_204,733	S—TAGGACAGCCAGTGAGGAGT A—TTCTCCTCTGCCACAGATGTC	99	S—Exon 3 A—Exons 4 & 5
LITAF	Lipopolysaccharide—induced TNF factor	NM_204,267	S—CTGTTCTATGACCGCCCAGT A—CTATGCACCCCAGCAGGAAGA	130	S—Exon 4 A—Exons 4 & 5
TGFB1	Transforming growth factor beta 1	NM_0,013,18456	S—CTGTACCAGGGTTACGGCAA A—CCCATCTCACAGGGACAGTG	174	S—Exon unknown A—Exon unknown
IFNB	Interferon, beta 1, fibroblast	NM_0,010,24836	S—CTCTTGCTTCTGCCAGCTCT A—CACGTCTTGTTGTGGGCAAG	141	S—Exon 1 A—Exon 1
IL1B	Interleukin 1, beta	NM_204,524	S—CTCACAGTCCTTCGACATCTTC A—TCACTTTCTGGCTGGAGGAG	123	S—Exon 4 A—Exons 4 & 5
IFNG	Interferon, gamma	NM_205,149	S—AAAGCCGCACATCAAACACA A—AGTCGTTCATCGGGAGCTTG	116	S—Exon 3 A—Exons 3 & 4
IL4	Interleukin 4	NM_0,010,07079	S—ATGACATCCAGGGAGAGGTTT A—TGCTCCACAATCCCTTTCTT	166	S—Exons 2 & 3 A—Exon 3
IL6	Interleukin 6 (interferon, beta 2)	NM_204,628	S—CTTCGACGAGGAGAAATGCC A—TAGCACAGAGACTCGACGTT	120	S—Exons 2 & 3 A—Exon 3
IL2	Interleukin 2	NM_204,153	S—ACCAACTGAGACCCAGGAGT A—CGGTGTGATTTAGACCCGTAAGA	170	S—Exons 2 & 3 A—Exons 3 & 4
IL12B	Interleukin 12B	NM_213,571	S—ACTACTGTCCATTTGCCGAAG A—GGTCTGGCTTTATGATATCTCTGA	121	S—Exon 4 A—Exons 4 & 5
IL17C	Interleukin 17C	XM_0,036,41945	S—AGCCTCACGAGAGATCCATC A—CCTCCCTGTCTTCACATCCAC	125	S—Exon 1 A—Exon 2
IL18	Interleukin 18	NM_204,608	S—AGTTGCTTGTGGTTCGTCCA A—TCCACTGCCAGATTTCACCTC	80	S—Exon 2 A—Exon 2 & 3
IL10	Interleukin 10	NM_0,010,04414	S—GAGTTTAAGGGGACCTTTGGC A—CTCTGCTGATGACTGGTGCT	107	S—Exons 2 & 3 A—Exon 3

S = Sense primer; A = Anti-sense primer. [1]primer sequences are provided in the interest of transparency but remain the intellectual property of qStandard (www.qstandard.co.uk)

mandatory gDNA wipe out step. The completed reaction was diluted 10-fold with 5 μg/mL tRNA in water.

Quantitative Real-Time PCR

Two microliters of cDNA were amplified in a 10 μL reaction using Agilent Brilliant III SYBR Ultra-Fast SYBR Green mix with each primer at a final concentration of 500 nmol/L. The no-template control reaction contained 2 μL of tRNA (0.5 μg/mL). DNA standards (10^7–10^1 copies/rxn) for each gene were included in each run. Reactions were pipetted robotically using a Qiagility (Qiagen, UK). Amplification parameters were: 95°C for 3 min followed by 40 cycles of 95°C for 5 s, 57°C for 1 s in a Rotor-Gene 6000. Melt curves were checked for product specificity (single peak) and the presence of primer dimers. All primers were designed to be intron-spanning so that any residual gDNA present could not be detected and avoided known SNP and secondary structures. Assays (Table 2) were designed by qStandard (www.qstandard.co.uk) and were tested for specificity by electrophoresis, efficiency > 95%, sensitivity to 10 copies/rxn, and linearity over 7 log by qPCR. Copy numbers/reaction were derived from the standard curves using the Rotor-Gene software. The 4 reference genes identified as the most stable using geNorm software (Vandesompele et al., 2002) were B2M, GAPDH, PPIA, and YWHAZ (Table 3). The normalization factor for each sample was determined using the method of Vandesompele et al. (2002) to normalize GOI copy numbers per reaction.

Analysis of Feed and Excreta

Dry matter (**DMR**) in feed and excreta was determined by drying of samples in a forced draft oven at 105°C to a constant weight (AOAC, 2005; method 930.15). Crude protein (6.25 × N) in samples was determined by the combustion method (AOAC, 2005; method 968.06) using a LECO FP-528 N (Leco Corp., St. Joseph, MI). Oil (as ether extract) was extracted with diethyl ether by the ether extraction method (AOAC, 2005; method 954.02) using a Soxtec system (Foss UK Ltd.). Gross energy (GE) values of samples were determined by an isoperibol oxygen bomb calorimeter (model 6200; Parr Instrument Co., Moline, IL) using benzoic acid as the reference material, following the manufacturer's recommendations.

Dietary MEn and Nutrient Retention

At the end of the feeding period, 2 birds from each pen were randomly selected and transferred to one of 40 wire-meshed metabolism pens (0.400 m^2 floor area) in a controlled environment room. The same diets were fed to the birds as they received in the feeding period. Feed and water were offered ad libitum. The birds were kept in the pens for 96 h, from 21 to 25 d age, and total excreta output was collected twice (every 48 h) from the trays beneath to avoid fermentation losses. Spilled feed and feathers were removed, and excreta were collected before weighing.

The coefficients for DMR, nitrogen (NR), and fat retention (FR) were calculated as the difference between the intake and the output of the respective nutrient and

Table 3. RT-qPCR assays[1] for reference gene selection for normalization of gene expression in *Gallus gallus* caecal tonsil tissue.

Gene symbol	Gene	Accession number	Primer sequences (5′-3′)	Product length, bp	Location
ACTB	Actin, beta	NM_205,518	S—TGACAATGGCTCCGGTATGTG A—CAACCATCACACCCTGATGTC	107	S—Exon 1 A—Exons 1 & 2
B2M	Beta - 2 microglobulin	NM_0,010,01750	S—GTACTCCGACATGTCCTTCAAG A—CACAGCTCAGAACTCGGGAT	157	S—Exon 2 A—Exons 2 & 3
GAPDH	Glyceraldehyde - 3- phosphate dehydrogenase Peptidylprolyl	NM_204,305	S—TGTGACTTCAATGGTGACAGC A—CCAAACTCATTGTCATACCAGGA	107	S—Exon 9 A—Exons 9 & 10
PPIA	isomerase A (cyclophilin A)	NM_0,011,66326	S—TGACAAGGTGCCCATAACAG A—CGTAAAGTCACCACCCTGAC	127	S—Exon 1 A—Exons 2 & 3
YWHAZ	Tyrosine 3 – monooxygenase/ tryptophan 5 – monooxygenase	NM_0,010,31343	S—TCTTGATCCCCAATGCTTCG A—TGCTCCACAATCCCTTTCTT	122	S—Exon 2 A—Exons 2 & 3

S = Sense primer; A = Anti-sense primer.
[1]Primer sequences are provided in the interest of transparency but remain the intellectual property of qStandard (www.qstandard.co.uk)

this was divided by the intake of the nutrient. Dietary MEn was calculated as described by Hill and Anderson (1958).

Statistical Analysis of Data

Data were statistically analyzed by two-way ANOVA using a 2 × 2 factorial randomized block arrangement of treatments. The main effects were the cereals (MC and WC) and additives (with and without PA) used. All data were analyzed with the ANOVA procedure of GenStat 15 statistical software package (IACR, Rothamstead, Hertfordshire, UK). In all instances, differences were reported as significant at $P < 0.05$.

RESULTS

No health problems were associated with use of cereal type or supplementary PA throughout the experiment. Mortality was low (<3%) and not treatment associated.

The relative expression (as copy numbers per reaction) of cytokine GOI in the caecal tonsils is presented in Table 4. Expression of IL2, IL18, IL10, and IL17C in the caecal tonsils were upregulated ($P < 0.05$) in the birds fed the MC-based diets. Dietary PA downregulated the expression of CD40LG ($P < 0.001$), IFNG, and IL6 ($P < 0.05$). There was a cereal type × PA interaction ($P < 0.05$) for IFNB and IL12B (Table 4). Expression of IFNB was downregulated in the birds fed PA supplemented MC-based diets, although expression of IL12B was downregulated in birds fed PA supplemented WC-based diet. The expression of LITAF, TGFB1, and IL1B was not influenced ($P > 0.05$) by dietary treatments. The expression of IL4 was undetectable for the majority of samples and therefore not included in the results table.

Results of growth performance variables, dietary MEn, and nutrient retention coefficients are presented in Table 5. Birds fed MC-based diets had 14.2% greater FI ($P < 0.001$), 4.6% greater MEn ($P < 0.05$), but 10.7% greater (less efficient) FCR ($P < 0.05$) compared to birds fed WC-based diets. The WG and nutrient retention coefficients were not affected ($P > 0.05$) by cereals used in diet formulations.

Feeding PA increased FI by 6.1% ($P < 0.05$), WG by 16.4% ($P < 0.001$), dietary MEn by 3.5% ($P < 0.05$), MEn: GE ratio by 2.7% ($P < 0.05$), dietary FD by 6.2% ($P < 0.05$), and reduced FCR (improved feed efficiency) by 9.4% ($P < 0.05$). There were no dietary cereal × PA interactions ($P > 0.05$) for any of the variables studied in Table 5.

DISCUSSION

Cytokines play a key role in both the adaptive and the innate immune system (Kim et al., 2010; Lillehoj et al., 2011). It has been speculated that the benefit of using PA in animal diets is associated with reduced intestinal inflammation resulting from a reduction of proinflamatory cytokines. Chao et al. (2008) reported that cinnamaldehyde suppressed the lipopolysaccharide-induced production of tumor necrosis factor (**TNF**), interleukin 6 (**IL6**) and IL1, thus suggesting that the inclusion of cinnamaldehyde could show suppressive effects on the production of various types of proinflammatory cytokines, which could explain the mitigation of the severity of coccidiosis when cinnamaldehyde is included in feed (Lee et al., 2010). Lee et al. (2013) also found that a mixture of capsicum and turmeric oleoresins was an effective phytonutrient against clinical signs of experimental avian necrotic enteritis when supplied in dietary form. Most cytokines have pleiotropic or redundant functions, and the level of one cytokine is tightly regulated by other cytokines. The overall picture tends towards an anti-inflammatory effect for the treatments supplemented with PA. The major proinflammatory cytokines studied in this case were IFNG, IL6, IL18, and IL1b. As the expression of both IFNG and IL6 cytokines were downregulated, this would indicate a lower inflammation level than in the other groups, under normal conditions (since no pathogen challenge was given). The LITAF is similar in that it regulates the expression of TNF-alpha (Hong et al., 2006). However, there are other regulators of TNF so further investigation into TNF expression may

Table 4. Relative expression (copies per reaction) of selected genes expressed in *Gallus gallus* caecal tonsil tissue.[1]

	Treatment factor[3]											
Item[2]	CD40LG	LITAF	TGFB1	IFNB	IL1B	IL12B	IFNG	IL2	IL18	IL6	IL10	IL17C
Diet												
W	230	15,576	84	8	56	83	29	3	206	25	6	2
M	222	14,064	99	12	47	75	24	9	289	24	18	6
PA												
–	282	15,900	103	12	50	95	33	5	232	38	10	5
+	170	13,740	80	9	53	63	20	7	263	11	14	3
SEM	14.8	1219.9	13.1	2.7	5.9	11.3	4.3	2.0	26.5	6.3	2.6	1.4
Diet and PA												
Wheat –	295	17,483	82	6[a]	54	122[a]	39	2	227	39	4	1
Wheat +	166	13,669	86	11[a,b]	59	44[b]	19	4	186	11	9	2
Maize –	269	14,318	125	18[b]	46	69[b]	27	8	237	37	17	9
Maize +	175	13,811	73	6[a]	48	82[a,b]	19	10	341	11	19	3
SEM	20.9	1725.2	18.5	3.8	8.4	15.9	6.0	2.8	37.5	8.9	3.6	2.0
Probabilities of statistical differences												
Diet	NS	NS	NS	NS	NS	NS	NS	0.037	0.039	NS	0.005	0.049
PA	<0.001	NS	NS	NS	NS	0.058	0.032	NS	NS	0.007	NS	NS
Diet × PA	NS	NS	NS	0.045	NS	0.009	NS	NS	0.068	NS	NS	0.070

[1]Based on the left caecal tonsil collected from 21 d old birds and stored in RNA*later*® (Sigma-Aldrich, USA) at –80°C prior analysis and 10 observations per treatment.

[2]W = wheat-based diet; M = maize-based diet; PA = supplemental phytogenic feed additive (100 g/t); (–) = diet was not supplemented with PA; (+) = diet was supplemented with PA.

[3]CD40LG = CD40 ligand; LITAF = lipopolysaccharide-induced tumor necrosis factor-alpha factor; TGFB1 = transforming growth factor, beta 1; IFNB = interferon beta; IL1B = interleukin 1 beta; IL12B = interleukin 12, subunit beta; IFNG = interferon gamma; IL2 = interleukin 2; IL18 = interleukin 18; IL6 = interleukin 6; IL10 = interleukin 10; IL17C = interleukin 17C.

Table 5. Broiler growth performance, dietary energy and nutrient retention.[1]

	Treatment factor[3]							
Item[2]	FI	WG	FCR	MEn	MEn:GE	DMR	NR	FR
Diet								
W	0.824	0.619	1.352	13.35	0.707	0.723	0.595	0.831
M	0.941	0.635	1.496	13.99	0.726	0.730	0.621	0.843
PA								
–	0.857	0.580	1.494	13.43	0.704	0.721	0.608	0.812
+	0.909	0.675	1.353	13.90	0.729	0.732	0.607	0.862
SEM	0.0114	0.0159	0.0329	0.144	0.0076	0.0091	0.0118	0.0141
Probabilities of statistical differences								
Diet	<0.001	NS	0.004	0.004	0.083	NS	NS	NS
PA	0.003	<0.001	0.005	0.031	0.031	NS	NS	0.019
Diet × PA	NS	NS	NS	NS	NS	NS	NS	NS

[1]Based on feeding period from 1 to 21 d of age for growth performance and from 21 to 25 d of age for ME, ME: GE, total tract DM retention coefficient, total tract N retention coefficient and total tract fat retention coefficient and 10 observations per treatment.

[2]W = wheat-based diet; M = maize-based diet; PA = supplemental phytogenic feed additive (100 g/t); (–) = diet was not supplemented with PA; (+) = diet was supplemented with PA.

[3]FI = feed intake (kg); WG = weight gain (kg); FCR = feed conversion ratio; MEn = dietary apparent metabolizable energy corrected for N retention (MJ/kg DM); GE = dietary gross energy; MEn: GE = metabolizability of dietary GE; DMR = total tract DM retention coefficient; NR = total tract N retention coefficient; FR = total tract fat retention coefficient.

be warranted, particularly as there were differences in the IL6 and IFNG between the groups. If all the groups were exposed to a bacterial challenge, it could explain why there are no differences in the LITAF expression levels between groups—LITAF is particularly responsive to bacterial products (LPS) so it would be much more difficult to observe differences in the expression of this gene under the current experimental conditions.

The other 2 genes (CD40LG and IL12p40) are expressed mainly by activated macrophages and dendritic cells, so these genes are a good indicator that an immune response is likely being generated (perhaps in response to the microbial loading provided by the recycle litter). These genes are also interesting in that they are involved in the activation of adaptive immunity (activation of Th1 cells and B cells) and it might be worth investigating other aspects of the adaptive immune response to check this.

The relatively low copy numbers of some GOI in this study, including IL4 (data not in tables), INFB, IL2, and IL10, suggests the results should be interpreted with caution as approaching the assay limit of detection may reduce the reliability of the data and

limit the ability to interpret the full profile of cytokine responses likely to be involved in a bacterial challenge (Reid et al., 2016). The production of proinflammatory cytokines would be expected in those macrophages where an inflammatory response occurs (Kaiser et al., 2000). In the present study, the majority of the cytokines showed significantly modulated expression in response to PA and/or diet type, thereby indicating their ability to modulate the innate immune response in the caecal tonsil tissue cells. Kim et al. (2010) also found that the local production of proinflammatory cytokines was significantly decreased when feeding the same mixture of PA to chickens.

In mammals, IL1B and IL6 are both critical for activating the immune response and synthesizing acute-phase proteins (Giansanti et al., 2006). It is speculated that these 2 proinflammatory cytokines might be essential in the early phase of the inflammatory responses.

The individual components in the mixture of PA, particularly capsicum, have been shown to have a protective function in the gastrointestinal mucosa increasing resistance to *Salmonella* colonization and organ invasion in broilers (McElroy et al., 1994). Karadas et al. (2014) reported an increase in hepatic antioxidants when the same PA blend was fed to broilers. Dhuley (1999) showed that carvacrol and cinnamaldehyde increase the activity of the antioxidant enzymes of the cells of the mucosa layer, which is known to be a protective system for the tissue. The latter could be the basis for the mucosal (villus-regulated) protective effect of PA previously observed by Jamroz et al. (2006). An improvement in health of the digestive mucosa by PA has also been demonstrated in piglets (Manzanilla et al., 2004).

Lee et al. (2010), reported that a combination of different phytonutrients (including capsaicin) promoted local protective immunity against avian coccidiosis caused by intestinal protozoan parasites, *Eimeria* spp., confirmed by increased levels of serum antibodies and increased levels of proinflammatory cytokine production in the duodenum. However, Lee et al. (2010) purposely challenged the birds with *E. acervulina*, thus severely provoking their protective immunity. In the present study, birds were placed on recycled litter only, and no specific challenge was applied, thus the measured copy numbers per reaction were relatively low. Rearing conditions influence the responses to PA (Pirgozliev et al., 2014), thus should be taken into account for a more complete interpretation of the experimental data emanating from experiments involving PA.

The growth performance results observed in the present study confirmed the growth-stimulating effect of the mixture of commercial PA that contains carvacrol, cinnamaldehyde, and capsicum oleoresin, in agreement with previous reports (Jamroz et al., 2003; Bravo et al., 2011). The relatively low weight of the birds compared to the breeders recommendations (Aviagen Ltd., Edinburgh, UK), was likely due to the 5% lower ME than commercial recommendations and feeding a mash diet, rather than a pelleted diet. For example, Pirgozliev et al. (2016) noted approximately 20% lower body weight in birds fed mash diets, compared to pellets.

Compared to MC, WC contains more water-soluble NSP, a carbohydrate complex with high water holding capacity, which may increase intestinal digesta viscosity, and reduce feed intake (Annison et al., 1996). Producing isoenergetic diets required more oil in WC-based diets formulation, which explains the greater MEn of them compared to the MC-based diets.

Feeding the mixture of phytogenics improved dietary MEn in accord with Mountzouris et al. (2010) and Bravo et al. (2011). As previously speculated (Bravo et al., 2011), the MEn effect of PA may be mediated by a direct increase in dietary energy availability, by a decrease in the energy required for the maintenance of the digestive tract, or a combination of both. This is supported by the increase in MEn: GE ratio (i.e., improved dietary energy utilization) in this study. Mitsch et al. (2004) also demonstrated that the same blend of PA reduced *Clostridium perfringens* colonization and proliferation in the gut of broilers, thus reducing the energy used by the bird to maintain the digestive tract ecosystem. In addition, Pirgozliev et al. (2015b) observed reduced heat production from broilers fed the same PA supplemented diets, speculating a reduced maintenance requirement of the birds.

In agreement with Hernández et al. (2004), FR was improved, further supporting the positive PA effect on available energy and growth performance variables. The reason for the enhanced availability with PA could be due to increased digestive secretions, as reported for the effect of capsicum oleoresin on pancreatic and intestinal enzyme activity in rats (Platel and Srinivasan, 2001), and on bile flow and bile acid secretion (Ganesh Bhat et al., 1984). Moreover, mixtures of spices exhibited an additive effect regarding their pancreatic enzyme stimulation compared with the spices taken individually (Platel et al., 2002).

The results show that supplementation of broiler chickens' diet with a standardized combination of 5% carvacrol, 3% cinnamaldehyde, and 2% capsicum oleoresin have beneficial effects on the performance as well as host immunity, possibly through immune modulation (reduced inflammation) of local effector cells in the gut. Since dietary phytochemicals influence gut microflora (Kim et al., 2015) in commercial broiler chickens, future studies to investigate the role of dietary phytochemicals on gut microbiota and immune system cross talks need to be carried out.

In conclusion, the present results indicate that a dietary combination of PA, including carvacrol, cinnamaldehyde, and capsicum oleoresin, improved the nutritional value of a WC- and MC-based diets fed to young broiler chickens. A reduction in CD40LG and IFNG indicate an anti-inflammatory effect of the PA mixture, suggesting an explanation for the

improvement in growth performance and MEn seen in the present study.

REFERENCES

Annison, G., R. J. Hughes, and M. Choct. 1996. Effects of enzyme supplementation on the nutritive value of dehulled lupins. Br. Poult. Sci. 37:157–172.

AOAC. 2005. Official Methods of Analysis. 18th ed. Association of Official Analytical Chemists, Washington, DC.

Bravo, D., P. Utterback, and C. M. Parsons. 2011. Evaluation of a mixture of carvacrol, cinnamaldehyde, and capsicum oleoresin for improving growth performance and metabolizable energy in broiler chicks fed corn and soybean meal. J. Appl. Poult. Res. 20:115–120.

Bravo, D., V. Pirgozliev, and S. P. Rose. 2014. A mixture of carvacrol, cinnamaldehyde, and capsicum oleoresin improves energy utilization and growth performance of broiler chickens fed maize-based diet. J. Anim. Sci. 92:1531–1536.

Chao, L. K., K. F. Hua, H. Y. Hsu, S. S. Cheng, I. F. Lin, C. J. Chen, S. T. Chen, and S. T. Chang. 2008. Cinnamaldehyde inhibits pro-inflammatory cytokines secretion from monocytes/macrophages through suppression of intracellular signaling. Food Chem. Toxicol. 46:220–231.

Dhuley, J. N. 1999. Anti-oxidant effects of cinnamon (Cinnamomum verum) bark and greater cardamon (Amomum subulatum) seeds in rats fed a high fat diet. Indian J. Exp. Biol. 37:238–242.

Ganesh Bhat, B., M. R. Srinivasan, and N. Chandrasekhara. 1984. Influence of curcumin and capsaicin on the composition and secretion of bile in rats. J. Food Sci. Technol. 21:225–227.

Giansanti, F., M. F. Giardi, and D. Botti. 2006. Avian Cytokines - An Overview. Curr. Pharm. Des. 12:3083–3099.

Hernández, F., J. Madrid, V. García, J. Orengo, and M. D. Megías. 2004. Influence of two plant extracts on broilers performance, digestibility, and digestive organ size. Poult. Sci. 83:169–174.

Hill, F. W., and D. L. Anderson. 1958. Comparison of metabolizable energy and productive energy determinations with growing chicks. J. Nutr. 64:587–603.

Hong, Y. H., H. S. Lillehoj, S. H. Lee, S. H. D. W Park, and E. P. Lillehoj. 2006. Molecular cloning and characterization of chicken lipopolysaccharide-induced TNF-α factor (LITAF). Dev. Comp. Immunol. 30:919–929.

Jamroz, D., J. Orda, C. Kamel, A. Wiliczkiewicz, T. Wertelecki, and I. Skorupinska. 2003. The influence of phytogenic extracts on performance, nutrient digestibility, carcass characteristics, and gut microbial status in broiler chickens. J. Anim. Feed Sci. 12:583–596.

Jamroz, D., T. Wertelecki, M. Houszka, and C. Kamel. 2006. Influence of diet type on the inclusion of plant origin active substances on morphological and histochemical characteristics of the stomach and jejunum walls in chicken. J. Anim. Physiol. Anim. Nutr. 90:255–268.

Kaiser, P., L. Rothwell, E. E. Galyov, P. A. Barrow, J. Burnside, and P. Wigley. 2000. Differential cytokine expression in avian cells in response to invasion by Salmonella typhimurium, Salmonella enteritidis and Salmonella gallinarum. Microbiol. 146:3217–3226.

Karadas, F., V. Pirgozliev, S. P. Rose, D. Dimitrov, O. Oduguwa, and D. Bravo. 2014. Dietary essential oils improve the hepatic antioxidative status of broiler chickens. Br. Poult. Sci. 55:329–334.

Kim, D. K., H. S. Lillehoj, S. H. Lee, S. I. Jang, and D. Bravo. 2010. High-throughput gene expression analysis of intestinal intraepithelial lymphocytes after oral feeding of carvacrol, cinnamaldehyde, or Capsicum oleoresin. Poult. Sci. 89:68–81.

Kim, J. E., H. S. Lillehoj, Y. H. Hong, G. B. Kim, S. H. Lee, E. P. Lillehoj, and D. Bravo. 2015. Dietary Capsicum and Curcuma longa oleoresins increase intestinal microbiome and necrotic enteritis in three commercial broiler breeds. Res. Vet. Sci. 102:150–158.

Kumar, S., S. B. Ciraci, P. Redmond, C. B. Chuammitri, D. P. Andreasen, and S. J. Lamont. 2011. Immune response gene expression in spleens of diverse chicken lines fed dietary immunomodulators. Poult. Sci. 90:1009–1013.

Lee, S. H., S. I. Jang, D. K. Kim, K. Ionescue, D. Bravo, and H. S. Lillehoj. 2010. Effect of dietary curcuma, capsicum, and lentinus, on enhancing local immunity against eimeria acervulina infection. J. Poult. Sci. 47:89–95.

Lee, S. H., H. S. Lillehoj, S. I. Jang, E. P. Lillehoj, W. Min, and D. M. Bravo. 2013. Dietary supplementation of young broiler chickens with Capsicum and turmeric oleoresins increases resistance to necrotic enteritis. Br. J. Nutr. 110:840–847.

Lillehoj, H. S., S. H. Lee, S. I. Jang, D. K. Kim, and K. W. Lee. 2011. Recent progress in understanding host mucosal response to avian coccidiosis and development of alternative strategies to mitigate the use of antibiotics in poultry production. Korean. J. Poult. Sci. 38:275–284.

Manzanilla, E. G., J. F. Perez, M. Martin, C. Kamel, F. Baucells, and J. Gasa. 2004. Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs1. J. Anim. Sci. 82:3210–3218.

McElroy, A. P., J. G. Manning, L. A. Jaeger, M. Taub, J. D. Williams, and B. M. Hargis. 1994. Effect of prolonged administration of dietary capsaicin on broiler growth and *Salmonella enteritidis* susceptibility. Avian Dis. 38:329–333.

Mitsch, P., K. Zitterl-Eglseer, B. Kohler, C. Gabler, R. Losa, and I. Zimpernik. 2004. The effect of two different blends of essential oil components on the proliferation of *Clostridium perfringens* in the intestines of broiler chickens. Poult. Sci. 83:669–675.

Mountzouris, K. C., V. Paraskevas, P. Tsirtsikos, I. Palamidi, T. Steiner, G. Schatzmayr, and K. Fegeros. 2011.Assessment of a phytogenic feed additive effect on broiler growth performance, nutrient digestibility and caecal microflora composition. Anim. Feed Sci. Technol. 168:223–231.

Munyaka, P., H. Echeverry, A. Yitbarek, G. Camelo-Jaimes, S. Sharif, W. Guenter, J. House, and J. Rodriguez-Lecompte. 2012. Local and systemic innate immunity in broiler chickens supplemented with yeast-derived carbohydrates. Poult. Sci. 91:2164–2172.

Oviedo-Rondón, E. O., M. E. Hume, C. Hernandez, and S. Clemente-Hernandez. 2006. Intestinal microbial ecology of broilers vaccinated and challenged with mixed Eimeria species, and supplemented with essential oil blends. Poult. Sci. 85:854–860.

Pirgozliev, V., D. Bravo, and S. P. Rose. 2014. Rearing conditions influence nutrient availability of plant extracts supplemented diets when fed to broiler chickens. J. Anim. Physiol. Anim. Nutr. 98:667–671.

Pirgozliev, V., D. Bravo, M. W. Mirza, and S. P. Rose. 2015. Growth performance and endogenous losses of broilers fed wheat-based diets with and without essential oils and xylanase supplementation. Poult. Sci. 94:1227–1232.

Pirgozliev, V., A. Beccaccia, S. P. Rose, and D. Bravo. 2015. Partitioning of dietary energy of chickens fed maize- or wheat-based diets with and without a commercial blend of phytogenic feed additives. J. Animal Sci. 93:1695–1702.

Pirgozliev, V., M. W. Mirza, and S. P. Rose. 2016. Does the effect of pelleting depend on the wheat sample when fed to chickens? Animal 10:571–577.

Platel, K., and K. Srinivasan. 2001. A study of the digestive stimulation action of select spices in experimental rats. J. Food. Sci. Technol. 38:358–361.

Platel, K., A. Rao, G. Saraswathi, and K. Srinivasan. 2002. Digestive stimulant action of three Indian spice mixes in experimental rats. Nahrung 46:394–398.

Reid, W. D., A. J. Close, S. Humphrey, G. Chaloner, L. Lacharme-Lora, L. Rothwell, P. Kaiser, N. J. Williams, T. J. Humphrey, P. Wigley, and S. P. Rushton. 2016. Cytokine responses in birds challenged with the human food-borne pathogen *Campylobacter jejuni* implies a Th17 response. R. Soc. Open Sci. 3:150541.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3:research0034–1.

Wallace, R. J., W. Oleszek, C. Franz, I. Hahn, K. H. C. Baser, A. Mathe, and K. Teichmann. 2010. Dietary plant bioactives for poultry health and productivity. Br. Poult. Sci. 51:461–487.

Windisch, W., K. Schedle, C. Plitzner, and A. Kroismayr. 2008. Use of phytogenic products as feed additives for swine and poultry. J. Anim. Sci. 86:E140–E148.